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The Effect of Presurgical Nasoalveolar Molding (Pnam) In Cleft Lip and Palate

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ABSTRACT

Purpose: To compare the bacterial flora in patients with cleft lip and palate (CLP) following presurgical nasoalveolar molding appliance (PNAM) therapy to (1) their pre-appliance flora, (2) the flora of their primary care provider, and (3) the flora of a healthy infant control patient population.

Materials & Methods: This is a case control blinded study of patients undergoing PNAM therapy at the UTHealth Post Doctorate Program in Pediatric Dentistry clinic aged birth to 6 months. The control patient population was derived from the UTHealth Physicians General Pediatric Clinic. Primary care providers were sampled at the initial visit only. Samples were obtained from the cleft group on the date of delivery of the appliance and the last visit prior to cheiloplasty. In the control infant group, the samples were obtained at their two-week well child check-up and four-month well child check- up. DNA was extracted from all samples and 16s rRNA gene sequencing was performed to identify the bacteria present in the sample. A differential abundance analysis was conducted to compare the difference in bacterial levels with a false discovery rate (FDR) corrected p-value of < 0.05 being significant.

Results: A total of six CLP patient-parent dyads were recruited for this study. Samples sites for the six parents and CLP patients were collected at the baseline. One hundred and seventy nine samples were collected and analyzed from the 12 dyads. Comparison of the collective infant group versus adult group revealed 918 operational taxonimic units (OTU's) identified with 390 OTU's (43%) noted to be significantly different.

Keywords: bacterial flora, cleft lip and palate (CLP), presurgical nasoalveolar molding appliance (PNAM) therapy

INTRODUCTION

Cleft lip and palate (CLP) are among the most common congenital anomalies and are collectively referred to as orofacial clefts. Cleft lip and/or palate is the second most common birth defect and the most common craniofacial anomaly [1]. The prevalence of oral clefts is estimated to be approximately 1.5 – 2 per 2,000 births [2] although racial and ethnic variations are present. Patients with CLP face years of surgical, dental, and speech intervention. Treatment of orofacial clefting may begin in the first week of life and can continue through adolescence depending on the severity of the defect. Treatment protocols may include presurgical infant orthopedic (PSIO) appliances, including pre-surgical nasoalveolar molding (PNAM) appliance, the Latham appliance, or lip taping.

The first resemblance of modern PSIOs appeared in the 1950's [3]. They are used to help position the alveolar segments in a closer proximity to facilitate the surgical correction of the cleft. Use of PSIOs can have other effects as well, such as the use of the PNAM appliance prior to surgical interventions to improve lip and nasal form, improve alar cartilage depression, projection of the nose tip, partially correct columella deviation, reduce the incidence of oronasal fistulas, and reduce the need for secondary bone grafting, especially if used in conjunction with gingivoperiosteoplasty [4-7].

Numerous studies have been completed investigating the caries prevalence in patients with oral clefts drawing inconsistent conclusions. While many studies concluded that patients with oral clefts had a higher prevalence of caries [1, 8-13] other studies have indicated that oral cleft patients have no significant difference in caries prevalence [13] or even a decreased prevalence of caries [14]. Recently it was shown in our CLP population, most of whom have undergone PNAM therapy, have an increase in dental caries [15]. This increase in caries prevalence in this population has been speculated by many to be due to insufficient dietary counseling, insufficient oral hygiene instruction, malposition of teeth, enamel hypoplasia, and poor accessibility of toothbrush to the teeth adjacent to the cleft [14]. Another explanation that could contribute to the increased caries risk in CLP patients may be that the PSIOs used to facilitate surgical repair may alter the bacterial flora of the oral cavity. Altering the oral environment can result in an acidic environment with decreased salivary flow on tissue surfaces in addition to inhibiting mechanical cleansing of the

oral cavity by the tongue [16]. The resin itself may promote plaque formation due to the roughness of the acrylic, resulting in an increased probability of accumulation of microorganisms [16]. Studies have demonstrated that children with oral clefts that wear acrylic appliances from shortly after birth were colonized earlier with *Mutans streptococci (*MS) and *Lactobacillus* (LB) than oral cleft patients without the acrylic appliance [17]. Colonization of the oral cavity with MS and LB is important, as earlier colonization with these microbes is associated with an increased incidence of caries [18].

Cariogenic microorganisms are acquired, and while there are other routes of transmission, numerous clinical studies have demonstrated that vertical transmission of bacteria from the mother is the primary source of MS for oral colonization of the child [19, 20].

The purpose of this study is to examine bacterial species of infants with and without CLP, to determine if the oral flora of patients with CLP is altered from healthy patients, and to determine if the use of an acrylic appliance changes the oral flora in these patients.

Additionally, we will compare the oral flora between infants with and without CLP and their primary caregivers. We hypothesize that while PSIO use significantly improves surgical outcome, it also changes the oral flora in patients with CLP compared to normal infants or their parents. The results of this study will help practitioner' s be aware of any changes the microbial flora present in the oral cavity of the predentate infant and may help develop oral hygiene protocols for these patients.

Methods and Materials

This study was approved by the Institutional Review Board of the University of Texas Health Science Center of Houston (UTHealth) (HSC-DB-16-0113). A case control blinded study was completed of patients that met the following inclusion criteria: (1) ASA I or ASA II classification, (2) patients aged zero to six months, (3) patients with CLP undergoing PNAM therapy at UTHealth Post Doctorate Program in Pediatric Dentistry Clinic or age- and insurance- matched controls receiving medical care at UTHealth Physicians General Pediatric Clinic, (4) primary caregiver willing to give their own microbial sample and willing to give informed consent for microbial testing of their child.

Patients with CLP were sampled twice: at the delivery of PNAM appliance and once PNAM therapy was complete. Age- and insurance-matched controls were obtained at the 2- week well child checkup and the 4-month well child checkup. Primary caregivers were sampled once, at the time their child was initially sampled. Microbial samples were obtained from the nostril, palate, maxillary buccal vestibule, and tongue of all subjects. Additionally, in the patients with CLP, samples were taken from the alveolar ridge and cleft space at both visits and the intaglio surface of the PNAM appliance at the last appointment only.

Collection of each specimen from the sample site was obtained using a microfiber sterile swab and the DNA was transferred to the DNA extraction and freezing solution. After collection of the specimens, the samples were stored at– 80°C in a secured freezer until analysis of all samples could be completed (not exceeding 12 months). The final volume of each sample is a standard 1.5 ml and the entire sample was consumed for bacterial DNA extraction.

Bacterial genomic DNA was extracted from specimens using the MO BIO PowerSoil DNA Isolation Kit following the manufacturer's protocol (MoBio, Carlsbad, CA). The 16S rRNA V4 region was PCR amplified and sequenced on the Illumina MiSeq platform by LC Biosciences (Houston, TX). All samples were processed and sequenced together. Amplification primers contained single-index barcodes resulting in PCR products that were pooled and sequenced directly. Reads were de-multiplexed based on barcodes and processed to remove poor quality sequence using CLC Genomics Workbench version 10 with the Microbial Genomics module. 16S rRNA gene sequences were assigned to specific operational taxonomic units (OTUs) at 98% identity by alignment to the Human Oral Microbiome (HOMD) database [21]. Analysis of microbiome communities was performed in CLC Genomics Workbench version 10. OTUs from the abundance table were aligned using MUSCLE with a required minimum abundance of 10 OTU's. Aligned OTUs were used to construct a phylogenetic tree using Maximum Likelihood Phylogeny using the Neighbor Joining method and the Jukes Cantor substitution model. Rarefication analysis was done by sub-sampling the OTU abundances in the different samples at a range of depths from one to 100,000; the number of different depths sampled was 20, with 100 replicates at each depth. Alpha diversity measures were calculated for observed OTUs, Chao 1-bias corrected, Shannon entropy and Simpsons Index. PERMANOVA Analysis (Permutational Multivariate Analysis Of Variance) was used to detect significant differences in Beta

diversity between groups, and comparisons were visualized using Principal Coordinate Analysis (PCoA). Differential abundance tests (non-parametric ANOVA) on the OTU frequency table were used to identify significant changes in the relative abundances of individual OTUs between groups. Differential abundance analysis was completed to compare mean bacterial species values and log fold changes between all groups. False discovery rate (FDR) correct p-values less than 0.05 were considered significant.

RESULTS

Subject population and demographics

Six CLP patient/parent dyads were recruited and initially sampled for this study; one patient was non-compliant with PNAM therapy and was not sampled with the post-NAM group. Six healthy age- and insurance-matched healthy controls were recruited for microbiome analysis. Demographic data for the patient and control groups are shown in Table 1, and sample sites per subject shown in Table 2. One hundred and seventy nine oral samples were collected and analyzed from the 12 dyads. Differential abundance analysis was performed on data from infant versus adult, infant with CLP versus healthy infant, and infant with CLP pre- versus post PNAM appliance.

Comparison of adult versus collective infant groups

Nine hundred and eighteen bacterial operational taxonomic units (OTUs), approximately equal to bacterial species, were identified within this sample set. Of these OTUs, 312 were found to be associated with bacteria from environmental habitats such as soil, water, and plant matter. The remaining 606 OTUs were associated with bacteria found in the human flora. When the cumulative infant microbiome from our subjects was compared to the adult microbiome of their parents, there were 390 (43%) OTUs that was significantly different (FDR p-value <0.05).

All of the significantly different species were further categorized as members of the normal oral flora, fecal species, bacteria derived from the environment, cariogenic bacteria, and known human skin and respiratory pathogens.

Comparison of healthy infant control with CLP group

When evaluating the CLP infants compared to the healthy infant group, 180 species total were identified. Of those, 131 species (72.8% of the OTUs) were significantly different.

In the CLP verses healthy infant group, nine (5%) pathogenic bacterial species were noted to be statistically significant including the environmental species *P. aeruginosa* (Figure 1). In the CLP population, the largest increases were seen in *H. influenza* (9.89 $\log_2$ difference), *P. aeruginosa* (8.85 $\log_2$ difference), *E. coli* (8.33 $\log_2$ difference), *K. pneumonia* (8.15 $\log_2$ difference), *H. aegyptius* (7.67 $\log_2$ difference), and *S. agalactiae* (6.23 $\log_2$ difference). In the healthy infant group, there were increased levels of *M. catarrhalis (*11.99 $\log_2$ difference), *D. pigrum* (5.32 $\log_2$ difference), *and S. pneumonia* (4.12 $\log_2$ difference)

Comparison of Appliance versus Cleft Space in CLP group

Operational taxonomic units (OTU's) were identified that were significantly different between the pre-surgical naso-alveolar molding appliance (PNAM) with the bacterial species identified in the cleft space, unaffected nostril, and the oral cavity of the CLP group. Fifty-nine samples were included in this analysis. Using differential abundance analysis, 148 species were identified as being significant (FDR p-value < 0.05).

In the appliance group versus the cleft space group, 17 (11%) species identified were statistically significant (Table 2). *P. aeruginosa, H. influenza, and H. parainfluenza* had the largest $\log_2$ fold difference of 12.9, 10.56, and 9.19, respectively (Figure 2) with the largest quantities in the cleft space.

In the appliance group versus the nasal group, 13 (8.8%) species were identified that were significant (Table 2). *H. parainfluenza, P. aeruginosa, and Actinomyces sp.* had the largest $\log_2$ fold difference of 10.2, 8.44, and 6.87, respectively with increased quantities seen in the nostril (Figure 1).

In the appliance group versus the oral group, 17 (11.5%) significantly different species were identified (Table 2). *Neisseria flavescens (*Log_2 fold difference of 11.37), *H. parainfluenzae* (Log_2 fold difference of 10.54), *Veillonella rogosae* (Log_2 fold difference of 10.02), and *Actinomyces sp.* (Log_2 fold difference of 6.76) had the largest quantities in the oral cavity. All other species identified in the appliance versus oral sites were noted to be in higher quantities on the appliance, most notably the species *Lactobacillus vaginalis* (Figure 3).

Comparison of sites in the CLP, healthy infant, and parent groups

Bacterial operational taxonomic units (OTU's) that were significantly different between each site were identified using differential abundance analysis (p-value <0.005). Oral sites included vestibular and palatal sample sites combined. Cleft space versus all other sites sampled was evaluated and presented in Table 2. The cleft space was not statistically significantly different when compared to other CLP sites (PNAM, tongue, unaffected nostril, oral sites). It was different from the healthy infant control group and parent sites. Oral soft tissue sites of the control child group were compared to all other sites. All sites were statistically different when comparing the healthy infant group versus all parent sites and CLP sites.

The parent oral sample sites obtained for the parent group were significantly different than the sites sampled from the CLP group including the cleft space, PNAM, and nostril sites (Table 2), as well as the healthy infant control group oral soft tissue and tongue sites. The oral sites of the parent groups were also significantly different from tongue samples in the parent groups (Table 1). All other parent sites compared to other parent sites were not statistically significant (comparison of tongue versus nasal sites and comparison of oral sites versus nasal sites).

Comparison of the oral soft tissue samples, tongue samples, and nasal samples in the healthy infant group versus the health infant group, were all found to be significantly different (Table 1). When evaluating CLP sites versus other CLP sites, the unaffected nostril was significantly different than the CLP tongue samples and the unaffected nostril of the CLP group was significantly different than the CLP oral soft tissue sites. The oral soft tissue sites obtained did not appear to be significantly different than the tongue (Table 1).

Table 1. Subject demographics

	Subject	Relationship	Gender	Race/Ethnicity
CLP subjects	**1.1**	Parent	Female	Hispanic
	1.2	Child	Female	Hispanic
	2.1	Parent	Female	Caucasian
	2.2	Child	Female	Caucasian
	3.1	Parent	Female	Caucasian
	3.2	Child	Female	Caucasian
	4.1	Parent	Male	Caucasian
	4.2	Child	Male	Caucasian
	5.1	Parent	Female	Cuban/Caucasian
	5.2	Child	Male	Cuban/Caucasian
	6.1	Parent	Female	Caucasian
	6.2	Child	Female	Caucasian
Controls	**11.1**	Parent	Female	Hispanic
	11.2	Child	Female	Hispanic
	18.1	Parent	Female	Hispanic
	18.2	Child	Male	Hispanic
	20.1	Parent	Female	Hispanic
	20.2	Child	Female	Hispanic
	22.1	Parent	Female	African American
	22.2	Child	Male	African American
	25.1	Parent	Female	Hispanic
	25.2	Child	Female	Hispanic
	27.1	Parent	Female	Caucasian
	27.2	Child	Female	Caucasian

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Table 2. Statistically significant environmental and pathogenic species identified in adult versus collective infant groups

Environmental and Pathogenic Species – Adult versus Child						
Bacterial Species	**Max group mean**	**Log_2 fold difference**	**Fold difference**	**P-value**	**FDR p-value**	**Bonferroni**
Pseudomonas aeruginosa	154.7058824	8.977216405	503.9778104	0	0	0
Staphylococcus aureus	41.91176471	5.217410427	37.20463415	4.05681E-11	5.17398E-10	3.72415E-08
Streptococcus agalactiae	8.764705882	5.796878062	55.59480072	4.8785E-09	4.39065E-08	4.47846E-06
Streptococcus pneumoniae	123.2058824	4.524020504	23.00731151	1.00065E-08	8.48346E-08	9.18597E-06
Streptococcus pyogenes	0.225490196	2.750147688	6.727860016	0.001807016	0.005967053	1
Mycobacterium tuberculosis	1.657142857	-4.292947778	-19.60225575	6.62692E-13	1.04888E-11	6.08351E-10
Moraxella catarrhalis	144.7254902	10.59275616	1544.320162	0	0	0
Dolosigranulum pigrum	156.8039216	9.512859455	730.5602536	0	0	0

Fig. 1. Log_2 fold difference of normal flora species identified in parent versus collective infant group

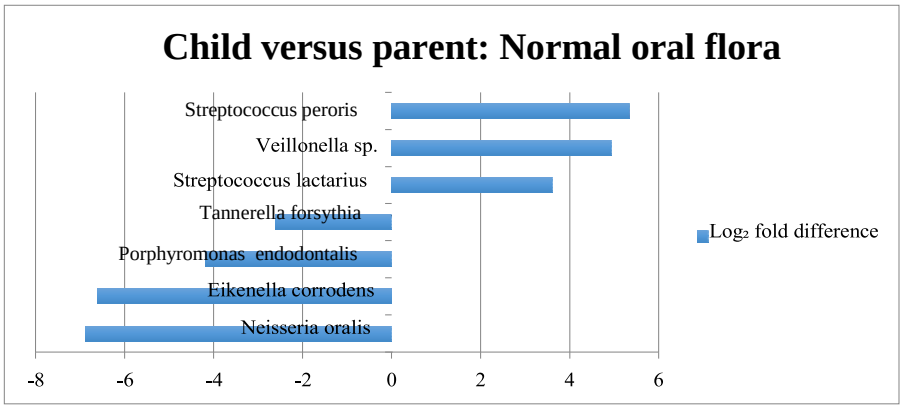


fig. 2. Log_2 fold difference of Lactobacillus sp. identified in parent versus collective infant groups

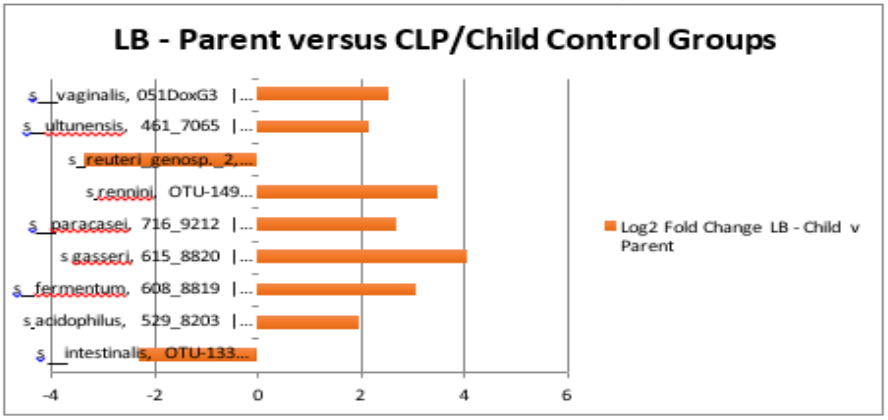
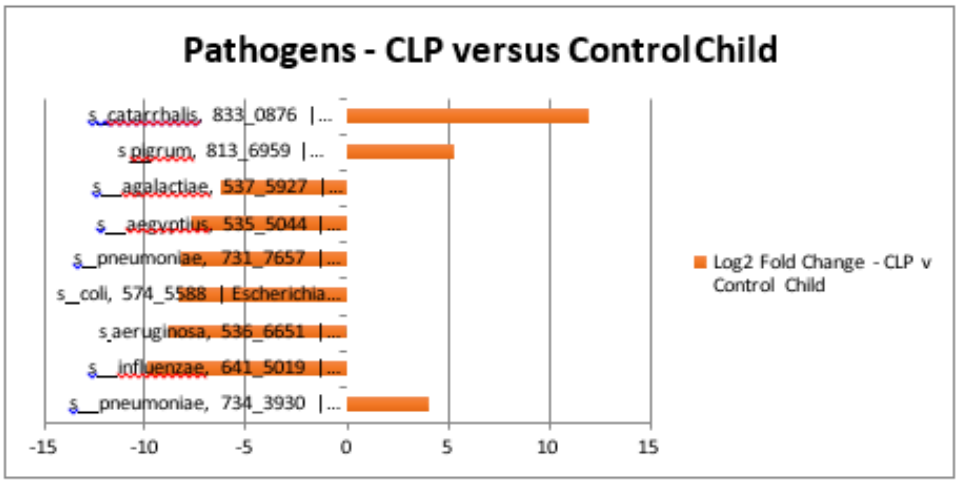


Fig. 3. Log_2 fold difference of pathogenic species identified in CLP versus healthy infant control group



Comparison of Parent-Infant Dyads

When evaluating bacterial flora by clusters, half of the parent-child dyads had overlapping communities within the adult cluster (Dyads 1, 2, 5, 18, 20, 25) while the other half did not (See fig. 18-29).

DISCUSSION

Normal flora identified in increased quantities in the infant group included S. peroris, Veillonella species, and S. lactarius, which is in agreement with other studies concluding that Streptococci, Staphylococci, Neisseria, Lactobacilli, Actinomyces, Veillonella, and Fusobacteria are the predominant bacterial species in the oral flora of infants [22-25]. Species such as S. peroris and Veillonella species have been identified as taxa that are early colonizers of the oral cavity. Our findings are similar to other studies suggesting the infant oral cavity contains the bacterial species necessary for biofilm and plaque development [25]. Species such as Tannerella forsythia noted in the adult population of our samples has been found in the sulcus of those with and without periodontal disease [26].

Eight percent of the statistically significant flora identified in the oral cavity of the infant population consisted of fecal species. This is in agreement with other studies noting that a significant portion of the oral flora in the early neonate period consists of transient intestinal microorganisms [27]. Identification of 61 statistically significant species of fecal flora was identified in the adult population. We hypothesize this may be related to changing the diapers of infants.

Multiple opportunistic pathogens were identified in the infant group including M. catarrhalis, D. pigrum, S. agalactiae, S. aureus, S. pneumonia, and S. pyogenes. On closer examination, the largest quantities of M. catarrhalis, D. pigrum, and S. pneumonia were identified in the healthy infant control group. While not noted in significantly higher concentrations in the CLP population M. catarrhalis in the CLP population has been identified in the cleft space prior to our study[28]. Other studies have noted higher fistula rates post-operatively in patients having positive swabs for M. catarrhalis [29].

Opportunistic pathogens specific to the CLP group included H. influenza, P. aeruginosa,

E. coli, K. pneumonia, H. aegyptius, and S. agalactiae. These findings are consistent with other studies noting opportunistic pathogens in the oral cavity of CLP subjects such as K. pneumonia,

S.agalactiae, and S. aureus [27]. A study by Roode et al. identified 23 pathogenic microorganisms from the soft palate and oro-nasopharynx of CLP patients prior to their cleft lip and palate repair. Similar opportunistic pathogens such as S. aureus, K. pneumoniae, H. influenza, and E. coli were identified in this study concluding that complete CLP patients presented with more pathogenic microorganisms prior to palatoplasty.

Group B β -hemolytic streptococcal species have not only been found in the CLP population, but have also been associated with higher risk of repair dehiscence [30]. The group B β-hemolytic streptococcal species S. agalactiae was identified in our CLP patient population only, and only at the

second time point. The highest quantities were identified in the palate but it was also identified in the cleft space, PNAM, tongue, and ridge.

While S. aureus was identified in all population, it was significantly higher in the collective infant population. The largest quantity was identified in the nostril of the healthy infant control. This is not surprising as previous literature has noted that S. aureus is most commonly found in the nares of humans [31]. A study completed in Brazil by Silva et al. identified S. aureus in the nostrils, mouth, and anus of newborns (Silva). Nelson-Filho et al. sampled 51 healthy newborns 8-10 hours after birth and identified S. aureus in 33.3% of newborns tested (Nelson-Filho). S. aureus was identified in the largest quantities in the cleft space and oral cavity of our CLP patients. Other studies have also noted a presence of S. aureus in CLP patient populations in the neonatal period, as well as prior to CLP patients prior to undergoing palate repair [23, 27, 30, 31].

As previously mentioned, the finding of Group B β-hemolytic streptococci in our CLP population is concerning, as well as the finding of S. aureus. Both have been associated with wound breakdown and failure of skin grafts [31]. The presence of β-hemolytic streptococci has been associated with delayed wound healing and the development of abscesses and fistulas in cleft lip and patient populations post-operatively [30]. Significant OTU's of S. agalactiae were identified in the CLP group at the second time point only in the cleft space and palate. This may suggest that S. agalactiae has an affinity for the cleft space.

K. pneumoniae was identified in large quantities in our CLP group when compared to the healthy infant control group and parent populations. This finding is an agreement with other studies noting the presence of K. pneumonia in CLP patients [27, 28, 31]. Traditionally, K. pneumoniae has been considered an opportunistic pathogen being a source of nosocomial infections [32, 33]. Recently hypervirulent and multidrug resistant strains have been identified resulting in severe and life-threatening infections in young and healthy individuals and is now considered a global health problem [33, 34]. All of the cleft lip and palate patients were colonized with K. pneumoniae at both time points sampled. The largest quantities of bacteria were noted in the CLP group in the cleft space at time point 1, time point 2, and the palate at time point 2. Increased levels in the cleft space at both time points with decreased levels noted in the healthy infant control group suggests that K. pneumoniae has an affinity for the cleft space.

Previous studies have noted pathogenic organisms similar to those found in our study [27, 28, 30, 31]. Debate exists between the need for prophylactic antibiotics prior to cleft lip and palate repair. Some recommend prophylactic antibiotics while others have recommended swabbing the oral cavity for pathogenic organisms prior to surgery and administered antibiotics as needed due to studies demonstrating large numbers of antibiotic resistant strains of opportunistic pathogens noted in this population [30, 31]. Roode et.al. identified strains of K. pneumonia in the CLP population that produced beta-lactamase and carbapenemase. Clinicians should appreciate that while an infant may appear healthy, it is possible that they are harboring potential pathogens that may contribute to postoperative infections and complications.

Lactobacillus species were noted to vary between the adult and infant groups. Of the identified strains of Lactobacillus occurring in increased quantities in the adult population, L. reuteri has been identified in carious lesions in adults [35]. Both L. acidophilus and L. fermentum were noted to be significantly higher in the infant groups with L. fermentum being significantly higher in the CLP group than the control child group. Both L. acidophilus and L. fermentum have been identified in children with caries [35, 36], with L. fermentum having been identified in children with early childhood caries [19, 37].

While no S. mutans was identified in our study, it has been suggested that S. mutans growth is promoted by lactobacillus [19, 38]. In contrast with our findings, previous studies have reported the presence of S. mutans near the time points sampled [19]. More recent studies have suggested that other taxa, identified in our collective infant population, such as Veillonella species may also contribute to early childhood caries [25].

No other studies to date have evaluated the difference in bacterial flora on the PNAM appliance in comparison to the cleft space, unaffected nostril, and oral sites (maxillary vestibule, palate, and alveolar ridge). When evaluating the cleft space, several species were identified in statistically significant quantities including P. aeruginosa, H. influenza, and H. parainfluenza in comparison to the appliance; H. parainfluenza, P. aeruginosa, and Actinomyces sp.in comparison to the appliance; and N. flavescens , H. parainfluenzae, Veillonella rogosae, and Actinomyces sp. with several of these being opportunistic pathogens. While no other studies were found to have evaluated the bacteria on the appliance, many of these species have been

identified in the cleft space such as H. influenzae and P. aeruginosa [28]. When evaluating the significantly different species most of the differences were found to be higher on the appliance. This may suggest that the appliance is harboring bacteria.

No other studies to date have evaluated the flora in the sites we evaluated in comparison to other sites sampled (comparison of all sites in all groups with all sites in all groups). The oral flora found in the cleft space was not significantly different when compared to the other CLP sites (PNAM, tongue, unaffected nostril, and oral sites). The sites sampled from the CLP group when compared to the adult and healthy infant populations were statistically significantly different. With an open communication between the nasal and oral cavity, we would expect to see the flora in the CLP to be different that the adult and healthy infant control groups as this creates a different bacterial environment.

In the parent group, there was a significant difference between the oral sample sites and tongue sample sites. This is not surprising as the tongue is highly keratinized and likely harbors different bacterium than the other oral sites [24].

In contrast to the CLP group, the comparison of all infant sites with other infant sites were significantly different. This may represent three distinct communities in the oral sites, tongue, and nostrils.

We noted that the oral sites of the parent were significantly different in comparison to the oral sites and tongue of the infant population. This may be expected as the adult microbiome is more established than the transient microbiome of the infant that does not contain a non-shedding surface for colonization.

In principle coordinate analysis of the parent-child dyads revealed that the infant sample sites at the two time points were not consistent in the bacterial communities identified. We also found that the sample sites did not appear to harbor bacteria similar to the same sample sites in the primary care provider. This may represent the difference between the transient bacterial communities noted in the infant population as compared to the established microbiome found in adult populations.

Limitations of our study included having a small sample size, limited comparable studies, and unclear birth history (vaginal versus cesarean section), which may have influenced the microbiome of the collective infant population.

CONCLUSION

Our study suggests:

- Several pathogenic bacterial species were identified that may have an affinity for the cleft space.
- The appliance harbored significantly more bacteria when compared to the nostril, cleft space, and oral cavity of the CLP group.
- While the microflora of the adult is established, the flora of the predentate infant is transient and different from adults.

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